REMARKS

Claims 87-140 were pending in this Office Action. The present response does not add or cancel any claims. Accordingly, it is claims 87-140, as presently amended, which are at issue.

The Office Action

In the Office Action mailed August 10, 2010, claims 87-140, all claims then pending, were rejected. Specifically, claims 87-92, 95-99, 101-102, 107-108, 110, 112, 114, 116-118, 120-127, 129-130 and 137-140 were rejected under 35 U.S.C. §103 over European Application 1 180 675 A2 of Hansen taken in view of U.S. Patent 6,280,961 of Kaplan as evidenced further by a publication of Smith.

In addition to the above mentioned base rejection, claims 93, 103, 110-111 and 114 were rejected under 35 U.S.C. §103 over the combination of Hansen and Kaplan and further in view of U.S. Patent 5,728,527 of Singer. Also, claim 94 was rejected under 35 U.S.C. §103 over the base combination of Hansen and Kaplan taken further in view of U.S. Patent 5,726,009 of Connors. Claims 100, 109, 113, 115 and 128 were rejected over the base Hansen/Kaplan combination taken further in view of U.S. Patent 5,428,451 of Lea. Claims 102, 104, 107 and 131-132 were rejected over the base Hansen/Kaplan combination taken further in view of U.S. Patent 5,877,161 of Riabowol. Claims 105, 107 and 135 were rejected under 35 U.S.C. §103(a) over the Hansen/Kaplan base combination taken further in view of U.S. Patent 5,691,147 of Draetta. Claims 106, 107 and 136 were rejected under 35 U.S.C. §103 over the Hansen/Kaplan base combination taken further in view of U.S. Patent 6,379,882 of Bitler. Claim 119 was rejected under 35 U.S.C. §103 over the Hansen/Kaplan base combination taken further in view of U.S. Patent 6,100,535 of Mathies. Claims 102, 104-105, 107, 131 and 133 were rejected under 35 U.S.C. §103 over the Hansen/Kaplan combination taken further in view of U.S. Patent

5,741,646 of Sherley. Claims 102, 104-105, 107 and 134 were rejected under 35 U.S.C. §103 as

being unpatentable over the Hansen/Kaplan combination taken further in view of U.S. Patent

5,344,760 of Harvey.

In addition, claims 87-140 were rejected under 35 U.S.C. §112, second paragraph, as

being indefinite with regard to the recitation of "the sample" in lines 18-19 of claim 87.

Claims 138 and 139 were objected to for allegedly including trademark terms therein.

Applicant thanks the Examiner for the Office Action and for the very thorough

explanation of the basis of the rejections.

The Rejections under 35 U.S.C. §112

By the present amendment, Applicant has revised claim 87 to delete "the sample" and to

replace it with the term "the mixed liquid material" which term has a proper antecedent basis.

Applicant thanks the Examiner for noting this informality.

In the present Office Action, the Examiner has maintained the rejection of claims 138 and

139 (noted as "claims 139 and 139" in the Office Action) on the basis that these claims include

trademark/trade names Cy3, Cy5, and Cy5.5. As Applicant explained in the response to the prior

Office Action, these terms are not trademarks or trade names but comprise standard

nomenclature for specific cyanine dyes as is well known in the art. In this regard, Applicant is

enclosing herewith a publication of Ernst et al., "Cyanine Dye Labeling Reagents for Sulfhydryl

Groups", *Cytometry* 10:3-10 (1989). This paper of Professor Ernst describes the synthesis and

use of a large group of cyanine dyes which may be utilized in the practice of the present

invention. This paper introduced the "Cy" nomenclature for these dyes and is well recognized in

the art. Consequently, Applicant respectfully submits that the terms utilized in claims 138 and

139 are specific to particular structures and would readily enable one of skill in the art to identify, synthesize, and use the particular molecular entities which this nomenclature refers to.

Applicant does acknowledge the fact that the Amersham corporation did sell particular versions of its Cy3 and Cy5 cyanine dye preparations under the corresponding "Cy3 Direct" and "Cy5 Direct" trademarks. These trademarks were registered; however, the "Cy3 Direct" registration has now lapsed. Applicant respectfully submits that the existence of these registrations in no way evidences that the species of cyanine dyes identified by the Cy nomenclature system in claims 138 and 139 constitute trademarks. It is very significant that the registrations for the Amersham preparations all included the word "Direct". These registrations were not granted for the Cy3 and Cy5 nomenclature itself. It is well established in trademark law that a proprietary trademark may include a generic term and still be registrable. A good example of this is "Coca Cola" which is undoubtedly the paramount trademark in the world at this time. That mark includes the generic term "cola" as is evidenced by the fact that numerous competitors are able to sell cola drinks without limitation. In the present case, the existence of the "Cy Direct" trademark registration in no way shows or suggests that the Cy nomenclature itself is a trademark. Reconsideration and withdrawal of this rejection is respectfully requested.

The Present Invention

Applicant will briefly recapitulate the principles and advantages of the present invention so as to better differentiate it over the prior art. The present invention relates to a method for assessment of particles in which a mixture comprising a liquid sample and a reagent agent may be obtained in a single step and directly transferred for data acquisition of intensities of electromagnetic signals, with subsequent analysis of said intensity data. The reagent material includes at least a first targeting species which is capable of selectively and directly binding to an

analyte position of said species of analytes said species of analytes having an amount of less than 1×10^6 analyte detectable positions per particle; the reagent further includes a labelling agent, wherein the first targeting species and the labelling agent are directly or indirectly coupled to each other. Said labelling agent is a compound capable of emitting, absorbing, attenuating or scattering electromagnetic radiation to result in the generation of a detectable electromagnetic signal.

The assessment of the sample is performed with an enlargement which is less than 1:20, and this is significant since it allows for the detection of a high number of particles which surprisingly can be separated from the background signal of unreacted labelling agent.

Prior art techniques for assessment of samples which contain the labelling agent require e.g. flow cytometry or a high degree of enlargement. Said enlargement is for example achieved by having a small amount of the sample fixated between a glass slide and a cover slip and studied by microscopy with sufficient enlargement to recognize each cell by its shape to distinguish it from the unreacted labelling agent still present. These assessments techniques are in direct contradiction with the present invention, by which a large sample volume can be assessed in one measurement and the labelled particles can be detected and distinguished from the unreacted labelling agent forming a background in the measurements, by the claimed processing of the intensities of the electromagnetic data. The inventors have found very surprisingly that this is still possible even though species of analytes labelled is present in an amount of less than 1×10^6 analyte detectable positions per particle as claimed. Such weakly labelled particles will, according to the hitherto accepted teachings of the prior art, otherwise require e.g. an advanced labelling technique, to allow easy detection.

The Rejections under 35 U.S.C. §103

None of the cited prior art alone or in combination teaches or hints the present method, as none of them differs from the accepted teachings that e.g. weakly labelled particles require special labelling in a multi step process, detection by microscopy and/or e.g. separation by fluorescence activated cell sorting (FACS). And thus, as it will be clear from the below the present invention as claimed is both novel and nonobvious in the light of the cited art, as there is no expectation of success based on general measurement techniques and the cited art.

D1 Hansen EP 1180675

Hansen relates to a method for assessment of at least one quantity or quality parameter of bacteria in a liquid material. As will be clear from the following several and significant differences between Hansen and the present invention exist.

Firstly the Examiner states that Hansen does not disclose that the number of analyte detectable positions is less than $1 \cdot 10^6$ as claimed by the present invention. Also the Examiner states that Hansen does not disclose that the first targeting species is coupled to the labelling agent. We agree on both of these points.

Further and equally important, Hansen does not teach that a liquid material (containing the particles/analytes of interest) is mixed with a reagent material at least comprising a first targeting species capable of selectively and directly binding to an analyte position ... wherein the first targeting species and a labelling agent are directly or indirectly linked to each other.

Even though the Examiner agrees that Hansen does not explicitly teach that the number of analyte detectable positions is less than $1 \cdot 10^6$, he argues that as the particles in both Hansen and the present application may be blood cells, the number of analyte detectable positions is less than $1 \cdot 10^6$ according to Hansen. However, the analyte detectible positions in each case are

different due to different conditions of the cells and the different labelling mechanisms. I.e. according to the teachings of Hansen the number of analyte detectable positions are $3 \cdot 10^9$ and not less than $1 \cdot 10^6$ as claimed in the present application. This is true as Hansen teaches core labelling and not labelling of "rare" analytes as essential in the present claims.

A cell contains a number of different species of analytes, which may be labelled in order to be able to detect specific properties which said cell may have. Different analytes are linked to different properties of the cell, and in e.g. a blood test, the properties which are investigated may related to analytes with analyte detectable positions (i.e. sites to which a targeting species may bind) which only are present in a low number pr. particle, even though a large number of analytes, with a large number of analyte detectible positions specific for other properties are present.

Thus, in the context of Hansen, due to the reagent material/process, the number of analyte detectable positions are $3 \cdot 10^9$, which leads to a significantly stronger signal from the labelling agent, traditionally allowing detection by methods not applicable in the case of weakly labelled particles. This is in contrast to the present invention in which a much weaker signal is to be detected due to the weak labelling of each particle.

Further, the Examiner assumes that the vaguely mentioned beads of Hansen are equal to the first targeting species of the present invention (Examiner's report p. 6, 1. paragraph). However, the beads according to Hansen are only mentioned once and as "polymer beads bound to biological particles or components in connection with assessment of these biological particles or components". Thus said beads bear no resemblance with the targeting species of the present invention as it is clear from the above discussion.

D2 Kaplan US 6,280,961

Kaplan relates to a method significantly different to both the one described by Hansen and in the present invention.

The present invention as claimed relates to a liquid material which is mixed with at least one reagent material, said reagent material at least comprising a first targeting species selectively and directly binding to an analyte position and a labelling agent. The first targeting species and labelling agent are directly or indirectly attached to each other in the reagent material. A volume of the liquid material (sample) comprising at least part of the mixture of the liquid material and the reagent material is then analysed. I.e. the results are achieved by a simple process.

According to Kaplan the sample containing the particles with the analytes to be detected is treated in a number of different steps as is clear from e.g. the examples of Kaplan. The detectable markers are added via a multi-step process which requires washing after each step and ends with a separation of the marked particles. This unambiguously teaches away from the present invention, as Kaplan teaches a method in which binding partners (targeting species) and detectable markers (labelling agent) are added to the particles of interest as separate components via separate steps in a complicated process.

Further, even if the separated particles, which is the end result of the method taught by Kaplan was to be examined by a technique similar to that of the present invention, the volume of liquid material (sample) would not contain *at least part of the mixture of the liquid material and the reagent material.*

According to the Examiner the first targeting species of the present invention is comparable to the magnetic particles of Kaplan (Examiner's report p. 6, last paragraph). However, the magnetic particles according to Kaplan are in fact detectable markers, i.e. it is

them which are actively used to facilitate the physical separation the method of Kaplan relates to. These detectable markers are added e.g. via a binding partner to either tyramide coated on a particle of interest (by a process involving a first binding partner) or as part of a molecule comprising tyramide attached to the detectable marker and a binding partner for binding to the first binding partner (col. line 48-58). Thus, the detectable markers are added in a late and separate step in the method for separation.

In Kaplan the first binding partner binds to the analyte of interest (col. 3 line 43-44, line 64-66) and is hereby to some degree comparable to the targeting species of the present invention. But there are significant differences, as the detectable marker is added to the binding partner (via several other elements) in a late step of a process involving multiple substances each containing e.g. different binding partners, tyramide or detectable markers. Hereby Kaplan unambiguously teaches away from the method according to the present invention in which it is a main point that a liquid material (containing the particle of interest) is mixed with a reagent material at least comprising a first targeting species capable of selectively and directly binding to an analyte position ... wherein the first targeting species and a labelling agent are directly or indirectly linked to each other. Also the use of multiple steps with different reagents and intermediate and final washing of Kaplan clearly teaches away from the present invention in that at least part of the mixture of the liquid material and the reagent material is arranged in a sample compartment as claimed by the present invention.

Also according to Kaplan the ability to detect particles with a low number of analytes depends on amplification of the labelling molecule and thus on the final number of detectable markers which can be attached to the particle. I.e. Kaplan's ability to handle a low number of analytes depends on increasing the signal by amplifying the number of detectable markers,

through a multistep method involving enzymatic activity of a second binding partner and a substrate with catalytic properties. This is in contrast to the present invention by which it is actually possible to detect a low number of attached labelling agents by the claimed combination of sample preparation, detection setup and data evaluation as described above.

Thus Kaplan teaches a laborious process while the present invention teaches a simple but yet effective method, which is very different from the ordinary teachings regarding detections of a low number of analyte detectable positions specifically a number less than $1 \cdot 10^6$.

Summary of Kaplan and Hansen

The method of Kaplan can be summarized to relate to physical separation of selected particles by use of a multistep process. In the method the particles are prepared with a first binding partner which binds to specific analytes of interest, subsequently the particles with the first binding partners are washed and put through a number of steps including second binding partners, tyramide, several times of washing and in the end the addition of a detectable marker which may be magnetic (iron oxide) or fluorescent. The particles may hereafter be separated e.g. in a magnetic field or by fluorescence activated cell sorting (FACS). In order to separate particles with a low number analytes, amplification steps are performed to increase the number of detectable markers attached.

The method of Hansen may include the step of colouring the particles for assessment by addition of one or more molecules, but it is not required as the particles may be analyzed basically "as is". The molecules may be added simultaneously or one at a time in which case measurements are performed in between addition. It is a goal of Hansen to limit the use of chemicals (molecules) in the process and that it is a simple process. Further the method of Hansen detects the particles in the sample preferably by detection of chemoluminescence,

photoluminescence, fluorescence or phosphorescence and is not a method of separation as Kaplan teaches.

It is not obvious at what stage the methods of Hansen and Kaplan should be combined to obtain the present invention. It does not make sense to combine Hansen and Kaplan in the colouring step of Hansen as this step according to Hansen preferably is quite simple and that Hansen by no means implies that an amplification of the signal (by the amplified labelling) or that physical separation is required here. If the methods are combined using Kaplan's method as a step for pre-treatment of the particles of Hansen the resulting method will still by no means comprise a simple labelling step using a targeting species directly or indirectly coupled to a labelling agent as according to the present invention.

In view of the foregoing amendments and remarks, Applicant respectfully submits that the present invention is novel and nonobvious with regard to the Kaplan and Hansen references taken either singly or in combination. Likewise, in view of the general inapplicability of the base rejection, further rejections on the grounds of the Hansen/Kaplan combination taken with further prior art are likewise overcome. However, Applicant will further discuss the merits of such secondary rejections hereinbelow.

Secondary References

Smith

The Smith publication teaches the use of flow cytometry and immunoprecipitation as assessment techniques which require labelling of cells. However, none of the mentioned techniques are relevant to either the claimed mixing of the sample of interest with a labelled targeting species or to the actual detection and data processing.

Singer (US 5,728,527)

Singer (US 5,728,527) relates to a method of nucleic acid hybridisation in living cells. It does not teach the detection of weakly labelled particles, it teaches a method which requires washing of samples before data acquisition as well as the cells to be viewed are contained on cover slips in a protective medium (Vectashield). The detection is achieved by modified or unmodified oligonucleotide probes.

Connors (US 5,726,009)

Connors teaches the use of a dye specific for viable cells BCECF-AM and a dead cell specific dye. Viable cells activate the fluorescence of BCECF-AM by esterases when BCECF-AM has penetrated the cell membrane of the live cell. The dead cell specific dye simply penetrates the cell membranes. The dead cell specific dye is propidium iodide *"Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA"* ref. *wikipedia.* None of these coloring methods are relevant in the light of the present invention. Further the dyed samples are investigated by microscopy.

Lea (US 5,428,451)

As discussed in an earlier reply Lea is not relevant in relation to the discussion of the present invention due to its use of flow cytometry. Further Lea teaches that lysing of magnetically labeled cells is preferred as well as a method which includes removal of unreacted labeling agent. The only dying agent mentioned is acredine orange which is comparable to propidium iodide in its mechanism and application.

Lea refers to PCT/EP90/02122 regarding magnetic particles. This document teaches the marking of cells with magnetic particles by a method requiring washing preferably more than

once. Dying is performed with acridine orange and includes a step of removing surplus dye. The dyed particles are then lysed.

Riabowol (US 5,877,161)

Mentions no relevant technique for labeling of a low number of analyte detectable positions specifically a number less than $1 \cdot 10^6$. Riabowol teaches the use of indirect immunofluorescence (ex 3 and 7), i.e. a technique requiring several steps in the labeling process. Further, the data relating to stained cells (e.g. fig 2A-2D) clearly shows the use of a microscopy technique with large enlargement. Thus Riabowol is not relevant to any of the steps of the present method.

Draetta (US 5,691,147)

Mentions detection of binding of particles of interest through a panning assay or by fluorescence microscopy of FARC, i.e. no relevant detection methods are taught (col 21 line 63 – col 22 line 10). Further no relevant technique for labeling of a low number of analyte detectable positions specifically a number less than $1 \cdot 10^6$ is mentioned, as well as Draetta is silent regarding the simple labelling procedure which is sufficient according to the present invention.

Bitler (US 6,379,882)

Teaches detection methods using Annexin V as dye. The use of Annexin V requires either flow cytometry or fluorescence microscopy, wherein labelled cells are arranged between a glass slide and a glass coverslip for assessment of the labelled particles.

Mathies (US 6,100,535)

Describes a confocal scanner and contains no relevant information on labelling techniques or detection by low (< 1:20) enlargement. Mathies is not relevant to the present main claim.

Sherley (US 5,741,646)

Labelling according to Sherley is not directed towards fluorescence detection but immunoprecipitations (col 8 line 43-60). I.e. the assessment method is not relevant in the context of the present invention.

Harvey (US 5,344,760)

Methods for detection as mentioned in col 12 line 52-66 all require a multistep processes for dying and assessment.

In view of the foregoing, Applicant respectfully submits that all claims are novel and nonobvious in view of the prior art of record taken either singly or in combination. Reconsideration and withdrawal thereof is respectfully requested.

Conclusion

In view of the amendments and remarks presented herein, all objections and rejections are overcome,and the application is in condition for allowance. Any questions, comments, or suggestions the Examiner may have which will place the application in still better condition for allowance should be directed to the undersigned attorney.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 07-1180.

Dated: Respectfully submitted,

By: /Ronald W. Citkowski/
Ronald W. Citkowski
Electronic Signature
    Registration No.: 31,005
GIFFORD, KRASS, SPRINKLE, ANDERSON
    & CITKOWSKI, P.C.
2701 Troy Center Drive, Suite 330
Post Office Box 7021
Troy, Michigan 48007-7021
(248) 647-6000
(248) 647-5210 (Fax)
Attorney for Applicant

Cyanine Dye Labeling Reagents for Sulfhydryl Groups[1]

Lauren A. Ernst, Ravinder K. Gupta,[2] Ratnakar B. Mujumdar, and Alan S. Waggoner[3]

Department of Biological Sciences and Center for Fluorescence Research, Carnegie-Mellon University,
Pittsburgh, Pennsylvania 15213 (L.A.E., R.B.M., A.S.W.) and Chemistry Department, Amherst College, Amherst,
Massachusetts 01002 (R.K.G., A.S.W.)

Cyanine and merocyanine dyes are introduced as new fluorescent reagents for covalently labeling proteins and other biomolecules. These dyes, which contain iodoacetamide functional groups, have high extinction coefficients and moderate quantum yields. A major advantage of these polymethine dyes is the easy manipulation of their spectral properties during synthesis. Cyanines containing reactive functional groups can be made with absorption maxima ranging from < 500 nm to > 750 nm. This property opens additional regions of the spectrum for experiments involving the simultaneous multicolor analysis of different fluorescent probes. The cyanines, which are relatively insensitive to solvent property changes, are complemented by the merocyanines, which are keen indicators of solvent polarity.

Key terms: Fluorescence, covalent labeling reagents, merocyanine dyes, fluorescent analog cytochemistry, red and infrared fluorescent probes

Fluorescence techniques have been used in a wide range of biological applications. The primary advantages of these methods are their high sensitivity and specificity and the ability to monitor events with high temporal resolution. When combined with microscopy, fluorescence methods are also capable of high spatial resolution. Since the detection of fluorescence is noninvasive, dynamic processes can be followed in living cells and organisms.

Fluorescent analog cytochemistry is a method developed to monitor the activity and distribution of specific cellular components in living cells (25). This technique involves covalently coupling a fluorescent probe to a purified native cellular component, such as a protein. The analog is characterized in vitro, incorporated into cells and then characterized in vivo. Many fluorescent analogs have already been prepared, including analogs of actin (26), tubulin (17), calmodulin (30), and phospholipids (21).

The variety and uses of fluorescent reagents capable of covalently labeling biomolecules have recently been reviewed (15). Waggoner discussed the ideal properties of fluorescent probes giving the maximum detectability of the probe with minimal perturbation of the function of the target molecule (28). Most of the reagents now available have absorption maxima at wavelengths less than 500 nm. The autofluorescence from normal cellular constituents is also highest in this spectral region (2). The amount of autofluorescence of a typical cultured fibroblast cell is equivalent to 35,000 molecules of fluorescein (19). Therefore, the detection of a fluoresceinated analog in these cells can be limited by the background 'noise.' It is also difficult to monitor simultaneously several different fluorescent parameters using the restricted selection of labeling reagents currently available (8).

The cyanine and merocyanine polymethine dyes have many of the properties presented as ideal for fluorescent probes. They have high extinction coefficients (> 100,000/mol cm) and moderate quantum yields and photostability. These dyes can be synthesized with excitation maxima ranging from about 500 nm to beyond 750 nm. Cyanine dyes (I, Fig. 1), are composed of two quaternized heteroaromatic bases (A and A') joined by a polymethine chain. These compounds have a cationic character due to the delocalized positive charge of the chromophore. The spectral properties of cyanine dyes are only slightly sensitive to solvent changes. Merocyanine dyes (II, Fig. 1), have a polymethine chain linking a quaternized basic nucleus (A) and a ketomethylene-derived nucleus (B). As demonstrated, merocyanines

[2]Ravinder K. Gupta's present address is Coulter Immunology, Inc., 440 W. 20th St., Hialeah, FL 33010.

[3]Address reprint requests to Dr. Alan Waggoner, Center for Fluorescence Research, Carnegie-Mellon University, Pittsburgh, PA 15213.
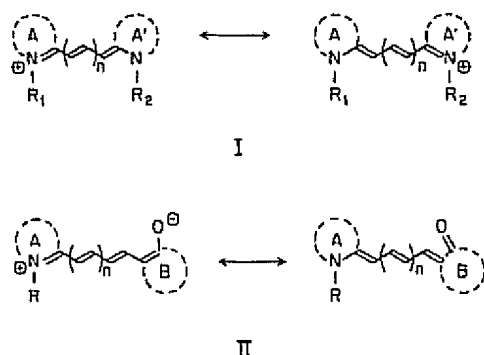
FIG. 1. Generalized structures of cyanine (I) and merocyanine (II) dyes. Notice that the cyanines contain a delocalized positive charge while the merocyanines can exist in both nonpolar and dipolar forms.

have both non-polar and zwitterionic resonance forms. The spectral properties of merocyanines are very sensitive to changes in solvent polarity. The syntheses and properties of these polymethine dyes have been well documented (10, 14). These dyes have previously been used to monitor membrane potentials (27) and to label lipid bilayers (20) and low density lipoprotein (3).

This is the first of a series of reports specifying the syntheses of cyanine and merocyanine dyes which are covalent labeling reagents for biological molecules. Our initial account describes cyanines and merocyanines containing iodoacetamido (IA) and iodoacetamidomethyl (IACH$_2$) groups which react rapidly and specifically with sulfhydryl groups at neutral pH. Although -SH groups are rare in extracellular proteins, these groups are common in proteins from the reducing environment inside cells. These new reactive polymethine dyes are therefore well-suited as fluorescent tagging reagents for fluorescent analog cytochemistry studies. The high selectivity of the iodoacetyl group for sulfhydryls allows easy handling of these dyes without loss of activity. Use of these reagents opens additional spectral windows allowing several analogs to be monitored with minimal interference by autofluorescence or resonance energy transfer.

MATERIALS AND METHODS
Spectroscopic Measurements

Absorption spectra were recorded on either a Perkin-Elmer Model 575 or a Varian Model 2200 spectrophotometer. Some fluorescence measurements were performed on a Hitachi Perkin-Elmer MPF-3 spectrofluorimeter equipped with either a Hamamatsu R-446 or R-928 photomultiplier tube. Other fluorescence measurements were obtained with a Spex Fluorolog-2 system. Infrared spectra were collected on a Nicolet Model 5DXB FT-IR spectrometer. Proton NMR spectra were taken on a Bruker 300 FT-NMR spectrometer.

Fluorescence Quantum Yield Determinations

The determinations of emission maxima and fluorescence intensities were carried out with dilute dye solutions exciting on a short-wavelength shoulder of the main absorption band. The absorbance of the sample was always less than 0.05. The fluorescence intensities were corrected for the intensity of the exciting light and the sensitivity of the detection system. The quantum yields were calculated relative to standard solutions in methanol of rhodamine 6G with ϕ = 0.95 (9) and a cyanine dye, 3,3'-diethyl-thiadicarbocyanine with ϕ = 0.33 (22). All sample dye solutions were made in 95% ethanol by diluting methanolic stock solutions.

Thin Layer Chromatography

Analytical thin layer chromatography of reactive dyes and their conjugates was performed on silica gel sheets, developed with ethanol-acetic acid (19:1), and on C18-reverse phase TLC plates developed with methanol-water (2:1).

Conjugation of Reactive Dyes

To a selected dye (~ 30 mg) dissolved in ethanol (6 ml) was added 3 ml aqueous cysteine (0.1M, pH 8). The reaction mixture was incubated for 20 min at room temperature, then concentrated by rotary evaporation to remove the alcohol. The resulting aqueous mixture was passed through a C18-Sep-Pak cartridge (Waters Associates, Milford, MA). After washing the cartridge with water (3 × 5 ml), the dye-cysteine conjugate was eluted with methanol. Evaporation of the methanol yielded almost pure conjugate (~ 25 mg).

Reaction of CY5.4-IA With F-Actin

The labeling of polymerized actin was achieved by a modification of the method of Wang and Taylor (29). Monomeric G-actin was dialyzed vs. ascorbate buffer to remove excess DTT, and then polymerized with high salt and magnesium ions. A 10-fold molar excess of dye (adsorbed at 10% w/w on celite) was added to the actin preparation. The mixture was stirred magnetically for 5 min and rocked gently for 30 min. After removal of the celite by a low speed spin, the labeling reaction was quenched by the addition of 2 mM cysteine. After a 15 min incubation the F-actin was sedimented by high speed centrifugation. The resulting dark blue pellet was resuspended in low salt buffer and depolymerized with potassium iodide. The actin (G-form) was purified by gel permeation chromatography over a G-25 Sephadex column, repolymerized and sedimented by high speed centrifugation. The recovered actin was depolymerized with low ionic strength Tris buffer and then dialyzed vs. Pipes injection buffer. The dye/protein ratio of the sample was measured, the sample was aliquoted, and then stored in liquid nitrogen until needed. An aliquot of this actin was assayed for polymerization ability following freezing. The biological activity of the cyanine-labeled

actin was tested by monitoring the incorporation of the protein into stress fibers after microinjection into swiss albino 3T3 cells (1). For observation of the cyanine labeled cells, the microscope was fitted with an excitation filter having a 30 nm bandpass centered at 620 nm and with an emission barrier filter having a 50 nm bandpass centered at 675 nm.

Preparation of Reactive Dyes

Procedures for the synthesis of representative dyes are given below. Overall yields for the multistep syntheses ranged from about 8% to 20% for the cyanine dyes and from 25% to 50% for the merocyanine dyes. Formation of the appropriate chromophore for each haloacetamido dye was indicated by its characteristic absorption maximum (5–7,24). The purified products had molar absorption coefficients and emission maxima comparable to unsubstituted model dyes synthesized in this laboratory. IR spectra of the haloacetyl-cyanines confirmed the formation of the cyanine chromophores. However the strong IR bands of the chromophores in most cases masked the transitions of the other functional groups. Even the amide I carbonyl stretching vibrations gave relatively weak bands. These unsymmetrical cyanines gave complex NMR spectra containing poorly resolved peaks which proved impractical for the characterization of the products. Although the reactive dyes showed only single components by thin layer chromatography, elemental analyses gave inconsistent results due to the presence of variable amounts of inorganic salts. These salts did not interfere with the reactivity of the dyes which was demonstrated by the conjugation reactions described above.

CY5.1-IA (V). 2,3,3-Trimethyl-(3H)-indole (1.6g, 10 mmol) and N-(3-bromopropyl)-phthalimide (2.7g, 10mmol) were heated at 110°C in a sealed tube for 4 h. The resulting red viscous mass, which solidified on cooling, was washed with ether (2 × 20 ml), then dried. The 1-(3-phthalimidopropyl)-2,3,3-trimethyl-indolinium bromide was refluxed for 5 h in 35 ml concentrated hydrochloric acid. The hydrolyzate was allowed to stand at room temperature for 2 d and the crystalline phthalic acid was filtered out. Basification of the filtrate with concentrated ammonium hydroxide caused a yellow precipitate to separate. The solid was collected, washed with water, and dried under vacuum. The dried residue was dissolved in dichloromethane (100 ml) and cooled on ice. Triethylamine (1.94 ml) was added followed by a solution of 0.57 ml chloroacetylchloride in dichloromethane (100 ml) during 1 h. The mixture was allowed to stand at room temperature overnight, washed with water (2 × 75 ml), dried over anhydrous sodium sulfate and concentrated to give 1.7g (~ 4.6mmol) of a light red semi-solid (III).

2,3,3-Trimethyl-(3H)-indole (0.48g, 3.0mmol) and 1,4-butanesultone (0.42g, 3.1mmol) were heated for 3 h in a stoppered flask at 120°C. The viscous red mass was cooled, washed with ether (2 × 5 ml), and dried under vacuum. The product was dissolved in 10 ml acetic anhydride, 0.77g (3.0mmol) of malonaldehyde dianilide hy-

drochloride (IV) was added (16), and the mixture was heated at 125°C for 30 min. The solution of anhydro 1-(4-sulfobutyl)-2-(4-acetanilino-1,3-butadienyl)-indolinium hydroxide (V) was cooled to room temperature and used without further purification.

The solution of anil (V) was mixed with 0.8g (2.1mmol) of the chloroacetamidoindoline (III). After the mixture was heated to 120°C for 20 min and then cooled on ice, it was diluted with ether (150 ml). The precipitate was collected and chromatographed over silica gel (85g). Elution with 30% ethanol in acetone yielded 0.64g (1.0mmol) of pure chloroacetamido dye, R_f 0.3 (acetone-ethanol 4:1). This dye (0.37g) was refluxed in methanol (80 ml) containing 0.75g (5mmol) sodium iodide for 4 h. The mixture was cooled on ice and then filtered. The filtrate was concentrated to dryness in vacuo. The residue was washed with chloroform (20 ml), acetone (3 × 2 ml), and hexanes (2 × 5 ml) and then dried. Pure CY5.1-IA (VI) was obtained, m.p. 190-193°C. IR (KBr) 925, 1,018, 1,041, 1,103, 1,141, 1,168, 1,338, 1,386, 1,455, 1,492, and 1,654 (amide I band) cm^{-1}.

CY5.6-IA (IX). 5-Chloroacetamidomethyl-1,3,3-trimethyl-2-methylene-indoline (VII) was prepared as described previously (13). 2-Methyl-benzothiazole (1.5g, 10mmol) and propane-1,3-sultone (1.5g, 12mmol) were heated at 120°C for 6 h. The resulting solid mass was triturated with ether, collected, and dried to give almost white crystals (2.55g, 9.4mmol). This material was mixed with 2.6g (10mmol) malonaldehyde dianilide hydrochloride (IV) and acetic anhydride (10 ml), then heated at 120°C for 30 min. The cooled reaction mixture was diluted with 50 ml ether and the ethereal layer decanted. The remaining dark red semi-solid mass was chromatographed over silica gel, eluting with a chloroform-ethanol step gradient. The product was recovered in 20% ethanol, yielding 1.6g (3.6mmol) of pure anhydro 1-(3-sulfopropyl)-2-(4-acetanilino-1,3-butadienyl)-benzothiazolium hydroxide (VIII).

The benzothiazolium anil (VIII) (0.4g, 0.9mmol) and 0.4g (1.4mmol) of the chloroacetamidomethyl-indoline (VII) were dissolved in 15 ml acetic anhydride and stirred for 30 min at room temperature followed by 2 min at 100°C. The resulting dye was precipitated with ether (30 ml) and purified by chromatography over silica gel. Elution with chloroform-ethanol (4:1) yielded pure chloroacetamidomethyl dye (0.1g, 0.2mmol). Refluxing this dye with methanolic sodium iodide as described above gave pure CY5.6-IA (IX). IR (KBr) 937, 1,017, 1,037, 1,106, 1,153, 1,321, 1,370, 1,459, 1,474, 1,508, and 1,656 (amide I band) cm^{-1}.

CY7.4-IA (XI). Butane-1,4-sultone (0.43g, 3.2mmol) and 2,3,3-trimethyl-(3H)-indole (0.5g, 3.1mmol) were heated for 3 h in a stoppered flask at 120°C. The resulting viscous mass was washed with ether (2 × 5 ml) and dried. To this material was added 0.85g (3.0mmol) of glutaconaldehyde dianilide hydrochloride (11) and acetic anhydride (10 ml). The mixture was heated at 120°C for 30 min and then cooled. This solution of anhydro 1-(4-sulfobutyl)-2-(6-acetanilino-1,3,5-hexatrienyl)-indolinium hydroxide (X) was used promptly without purification.
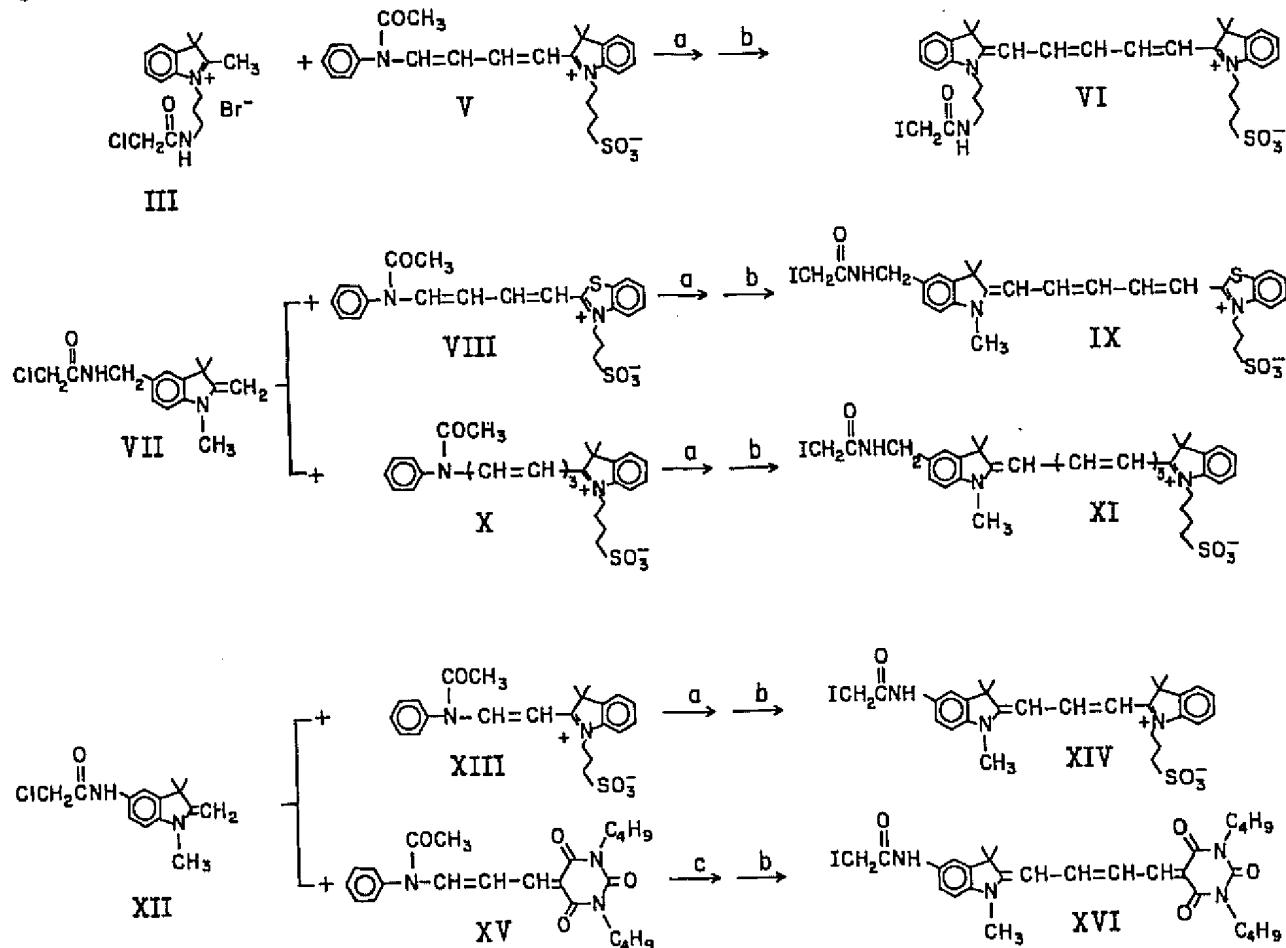
FIG. 3. Synthetic schemes for representative iodoacetamido polymethine dyes. a, acetic anhydride; b, NaI/methanol; c, methanol.
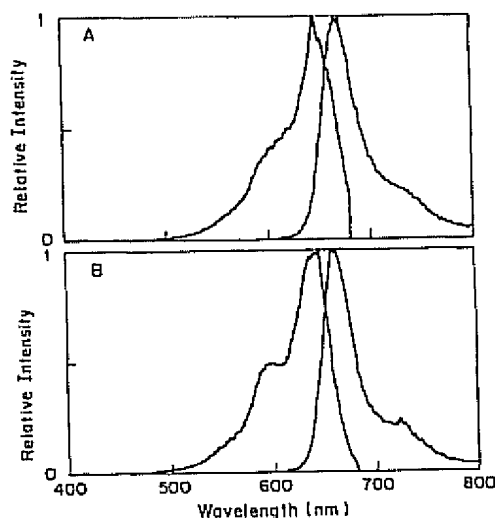

FIG. 4. Excitation (left) and emission (right) spectra for a) CY5.4-G-actin in Buffer E (29) and b) free CY5.4-IA in Buffer E. The excitation spectra were collected by monitoring the emission intensity at 690nm. The emission spectra were obtained by exciting at 600nm. The spectral bandpass of both monochromators was 1 nm.

Polymethine dyes containing different terminal heterocyclic groups (Y = O, S, C(CH$_3$)$_2$) exhibit different absorption and fluorescence maxima, although the effect is smaller than that observed with the vinylene homologs. The greatest differences are seen between oxygen heterocycles and sulfur heterocycles. The cyanines CY5.5-IA and CY5.6-IA show a 24 nm difference while the merocyanines MC4.1-IA and MC4.2-IA exhibit a 40 nm difference. The dependence of spectra on the terminal heterocycle enables manipulation of the absorption maxima in probes designed for specific applications. The spectral properties of probes can be fine tuned to give maximal overlap for resonance energy transfer experiments or to give minimal overlap between probes in multiple parameter experiments. Other characteristics of these dyes depend on Y. For example, indolinyl cyanines show a greater photostability than other similar cyanines (24). In our experience benzothiazolyl cyanines are more susceptible to oxidative decomposition, both photolytic and chemical, than the benzoxazolyl dyes, and the oxygen containing heterocycles generally have larger quantum yields than the sulfur containing heterocycles.

A solution of 1.1g (3.9mmol) chloroacetamidomethyl indoline (VII) in 5 ml acetic anhydride was added to the solution of the anil (X), and the mixture was heated for 30 min at 120°C. The product was precipitated by the addition of ether (70 ml). The oily residue was twice dissolved in ethanol (5 ml) and reprecipitated with ether (30 ml). The resulting oil was chromatographed over silica gel with a chloroform-ethanol step gradient elution. The desired product eluted in 20% ethanol. Concentration of the appropriate fractions gave 0.35g (0.55mmol) of chloroacetamido dye. Treatment of this product (0.32g, 0.50mmol) with sodium iodide (0.19g, 1.3mmol) in boiling methanol as previously described yielded 0.35g (0.48mmol) of CY7.4-IA (XI). IR (KBr) 925, 999, 1,032, 1,085, 1,097, 1,138, 1,412, 1,447, 1,515, 1,541, and 1,656 (amide I band) cm^{-1}.

CY3.2-IA (XIV). 5-Chloroacetylamino-indoline (XII) was prepared by the method of Gale and Wilshire (12.) Propane-1,3-sultone (1.3g, 11mmol) and 2,3,3-trimethyl-(3H)-indole (1.7g, 11mmol) were heated at 100°C for 3 h. The mixture, which solidified on cooling, was washed with ether (2 × 5 ml) and dried in vacuo. To this solid was added 2.0g (10mmol) of N,N'-diphenylformamidine and acetic anhydride (12 ml). The mixture was heated at 120°C for 30 min, cooled, and diluted with ether (30 ml). The solid product was collected and crystallized from ethanol-ether giving 1.5g (3.5mmol) of the anil (XIII), m.p. 308–310°C.

The chloroacetamido indoline (XII) (0.8g, 3.0mmol) and the anil (XIII) (1.3g, 3.1mmol) were dissolved in 15 ml acetic anhydride, heated for 10 min at 120°C, then cooled to room temperature. The reaction mixture was diluted with ether (40 ml). The red solid was collected and dried yielding 1.6g (~ 2.5mmol) of almost pure chloroacetamido dye. This dye (1.4g, ~ 2.2mmol) was refluxed for 2.5 h in methanol (150 ml) containing 1.4g (9.3mmol) sodium iodide. After concentration of the reaction mixture to 10 ml, the product was precipitated by the addition of acetone (200 ml) to give 1.7g of crude product. Chromatography over silica gel with ethanol-chloroform (3:7) elution yielded 0.8g (1.2mmol) of pure CY3.2-IA (XIV), m.p. 245–250°C, R$_f$ 0.37 (acetonitrile-ethanol 2:1). IR (KBr) 929, 1,035, 1,115, 1,152, 1,215, 1,370, 1,415, 1,458, 1,475, 1,560, and 1,680 (amide I band) cm^{-1}.

MC4.1-IA (XVI). 1,3-Dibutyl-barbituric acid was synthesized from 1,3- di-n-butyl urea and diethylmalonate by the method of Brooker et al. (4.) 12g (50mmol) of the barbituric acid, 13g (50mmol) of malonaldehyde dianilide hydrochloride (IV), and 4.1g (50mmol) of sodium acetate were refluxed in acetic anhydride (75 ml) for 20 min. The reaction was cooled to 0°C and a mixture of ice and methanol was added until a precipitate began to form. The precipitate was collected, washed with cold methanol, and dried giving 12.8g (~ 31mmol) of brownish yellow anil (XV).

0.53g (2.0mmol) of the chloroacetamido indoline (XII) and 0.83g (2.0mmol) of the barbituric acid anil (XV) were stirred in methanol at room temperature for 20 h. The mixture was concentrated and chromatographed over silica gel. Elution with chloroform produced 0.81g

(1.5mmol) of the chloroacetamido dye. As with the other dyes, treatment with sodium iodide in boiling methanol gave the iodoacetamide, MC4.1-IA, XVI, m.p. 76–78 °C. IR (KBr) 999, 1,100, 1,169, 1,185, 1,312, 1,358, 1,408, 1,458, 1,474, 1,643, and 1,702 (amide I band) cm^{-1}.

RESULTS AND DISCUSSION
Nomenclature and Structure of New Dyes

A wide variety of cyanine and merocyanine dyes can be synthesized by incorporating various substituents in structures Ia and IIa at positions X, Y, and R$_1$ to R$_3$ (Fig. 2). To minimize the possible confusion we will name the dyes using the format, XX#.***-ZZ. In these names XX represents the type of polymethine dye (14): CY, for cyanines; MC, for merocyanines; OX, for oxonols; ST, for styryls; PY, for pyryliums and so on. The # term gives the number of carbon atoms in the polymethine bridge (e.g., # is 3 when m=1 and # is 5 when m=2). the ZZ term refers to the active functional group attached to the dye moiety. In this paper ZZ is IA for iodoacetamide and IACH$_2$ for iodoacetamidomethyl, but other reactive groups will also be incorporated into the dye structures, including maleimide (MAL), isothiocyanate (ITC), N-hydroxysuccinimide ester (OSU), dichlorotriazinylamine (DCT), and azide (AZ) to list but a few. When the dyes are covalently attached to a protein, the ZZ term can define the conjugate. The *** term will be a number unique for each combination of substituents in the polymethine structure, X, Y, and R$_1$ to R$_3$. This naming system for the reactive polymethine dyes will simplify referencing the many combinations of substituents possible.

Two types of polymethine dyes were synthesized in this study. A haloacetamido group was incorporated into the cyanines, Ia (Fig. 2), and merocyanines, IIa (Fig. 2), at positions R$_1$ or R$_3$. To enhance the water solubility of the cyanines, R$_2$ was an alkyl sulfonic acid. In the merocyanines, R$_2$ was a short chain alkyl group which resulted in only limited solubility in aqueous solvents for these dyes.

Synthesis of Dyes

The synthetic schemes for representative dyes are shown in Figure 3. Standard procedures were used for the synthesis of unsymmetrical cyanine and merocyanine dyes (14). The main reactions involved formation of substituted acetanilides from quaternized heterocyclic intermediates and dialdehyde anils. These ani-
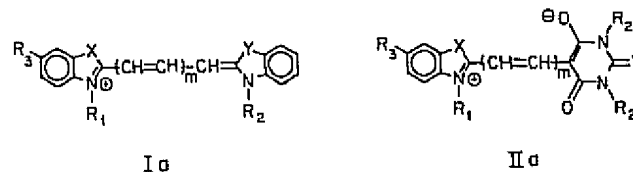

FIG. 2. Structures of the cyanine (Ia) and merocyanine (IIa) dyes synthesized in this study. The various substituents of individual dyes are given in Table 1.

Table 1
Structural and Spectroscopic Properties of New Reactive Dyes

Dye no.	\multicolumn{6}{c}{Structures[a]}	\multicolumn{4}{c}{Properties[b]}
	M	X	Y	R_1	R_2	R_3	Abs.max (nm)	ϵ max ($\times 10^{-3}$)	Fluor. max.(nm)	Q.Y.
Cyanines										
CY5.1-IA	2	I	I	IAC_3H_6	C_4SO_3	H	650	147	668	0.40
CY3.2-IA	1	I	I	C_1	C_3SO_3	IA	565	116	590	0.07
CY5.3-IA	2	I	I	C_1	C_4SO_3	IA	662	197	689	0.12
CY5.4-IA	2	I	I	C_1	C_4SO_3	$IACH_2$	652	128	669	0.27
CY5.5-IA	2	I	O	C_1	C_3SO_3	$IACH_2$	611	139	637	0.42
CY5.6-IA	2	I	S	C_1	C_3SO_3	$IACH_2$	651	125	674	0.34
CY5.7-IA	2	I	S	C_1	C_3SO_3	IA	662	124	690	0.17
CY7.4-IA	3	I	I	C_1	C_3SO_3	$IACH_2$	752	195	773	—
Merocyanines										
MC4.1-IA	2	I	O	C_1	C_4	IA	582	119	607	0.08
MC4.2-IA	2	I	S	C_1	C_2	IA	606	155	628	0.05
MC6.1-IA	3	I	O	C_1	C_4	IA	683	78	712	0.14
MC6.2-IA	3	I	S	C_1	C_2	IA	705	175	728	0.08

[a]Structural variables relate to general structures Ia and IIa. I = isopropylidene, C_1 = methyl, C_2 = ethyl, C_4 = butyl, C_3SO_3 = sulfopropyl, C_4SO_3 = sulfobutyl, IA = iodoacetamide (ICH_2CONH-).
[b]All spectral measurements used 95% ethanol as solvent. Quantum yields of the dyes on labeled proteins may be significantly different.

Table 2
Spectral and Chromatographic Properties of Selected Reactive Dyes and Their Cysteine Conjugates

Reactive dye no.	\multicolumn{2}{c}{Absorption maximum[a]}	\multicolumn{2}{c}{R (silica gel TLC)[b]}	\multicolumn{2}{c}{R (C_{18}-RPTLC)[c]}
	Dye	Conj.	Dye	Conj.	Dye	Conj.
CY3.2-IA	565	564	.38	.05	.50	.66
CY5.6-IA	646	647	.36	.02	.56	.60
CY5.1-IA	646	646	.45	.03	.40	.51
CY7.4-IA	749	750	.40	.05	.42	.53
MC4.1-IA	582	582	.79	.19	.22	.31

[a]Solvent was 95% ethanol.
[b]Developing solvent was ethanol-acetic acid (19:1).
[c]Developing solvent was methanol-chloroform (2:1).

lides were then condensed with a chloroacetamide containing indoline to form the dye chromophore. The dyes were converted to the more reactive iodo derivatives using methanolic sodium iodide.

Three methods were used to link the haloacetyl group to the indoline nucleus. Quaternization of 2,3,3-trimethyl-(3H)-indole with a protected alkyl amine was the most straight forward approach (CY5.1-IA) method). Electrophilic substitution of the indoline using procedures developed by Gale and coworkers placed the acetamido group directly on the aromatic nucleus (12) or formed an acetamido-methyl substituent in which the amide group is not conjugated with the aromatic system (13).

A variety of different anilides were used in these dye syntheses. The basic heterocycles 2-methyl-benzoxazole, 2-methyl-benzothiazole, and 2,3,3-trimethyl-(3H)-indole

quaternized with either 1,3-propanesultone or 1,4-butanesultone formed cyanine dyes. 1,3-Dialkyl-barbituric acid and 1,3-dialkyl-2-thio-barbituric acid formed merocyanines. Dyes with the heterocyclic nuclei connected by vinylene chains of varying lengths were made using the vinylogous series N,N'-diphenyl formamidine, malonaldehyde dianilide, and glutaconaldehyde dianilide. The specific structures and the spectral properties of the new polymethine dyes are given in Figure 2 and Table 1. The polymethine syntheses are versatile. Many other combinations of heterocyclic nuclei and dianils can be used to form dyes of these types.

Properties of Dyes

Cyanine and merocyanine polymethine dyes are characterised by sharp, intense absorption bands (ϵ > 100,000) and corresponding sharp emission profiles with Stokes shifts ranging from 15 nm to 25 nm. While the quantum yield of fluorescence for these dyes is typically between 0.2 and 0.4 in ethanol, we have found that their fluorescence decreases by a factor of about three in water. The absorption properties of these dyes are primarily determined by three structural features, m, Y, and R_3 in structures Ia and IIa. When the polymethine chain is lengthened by one vinylene unit (m to m+1), the absorption maximum is shifted to longer wavelength by about 100 nm. This relationship allows the synthesis of probes with very similar structural features that are excited in different regions of the spectrum. Thus, CY3.2-IA (m = 1) and CY5.3-IA (m = 2) have similar solubilities and reactivites, but have absorption maxima at 565 nm and 662 nm, respectively. Other examples of this characteristic can be found in Table 1 for both the cyanine and merocyanine dyes.

Substituents on the aromatic portion of the cyanines, R_3 in structure Ia, also affect the spectral properties of the dyes. For the substituents investigated here, alkyl substituents (R_3 = IACH$_2$) shift the spectra only slightly compared to the unsubstituted nucleus (R_3 = H). (For example, compare CY5.4-IA to CY5.1-IA.) However, conjugation of the amide with the chromophore (R_3 = IA) causes red shifts of about 10 nm in the absorption maxima and 15 nm to 25 nm in the emission spectra. (Compare CY5.7-IA (R_3 = IA) with CY5.6-IA (R_3 = IACH$_2$). The site and manner of iodoacetamide group attachment to the cyanines greatly affects the efficiency of fluorescence of these dyes. Substitutions on the chromophore ring systems decrease the observed quantum yields by as much as 70% when the amide is linked directly with the chromophore.

The synthetic flexibility of the cyanine dyes is very useful when designing new fluorophores. The excitation and emission peaks of the dyes can be selected to occur in specific regions of the spectrum. This property is important in laser illumination based systems, in experiments involving several fluorophores used together and in energy transfer experiments. Other characteristics of the cyanine dye intermediates can be used in the design of probes for specific applications. Dyes with enhanced or decreased chemical or photo-stability can be made. Symmetrical cyanine dyes containing two reactive groups can also be synthesized. The capacity of these bifunctional dyes to act as crosslinking reagents could be used to advantage. In rotational mobility studies it is sometimes desirable for the rotation of the chromophore to represent the rotational mobility of the biological macromolecule to which it is attached. Linking both ends of the fluorescent probe to the macromolecule would limit the independent movement of the label.

Reactions of Dyes

The reactivity of these iodoacetamido dyes was verified by the facile formation of dye-cysteine conjugates. The coupling reactions were complete in less than 20 min at pH 8 and gave single products with the dyes tested. Under these conditions iodoacetyl derivatives have high selectivity for sulfhydryl groups (18), which was indicated by the formation of only one product from each reactive dye. Absorption and chromatographic properties of selected dyes and their cysteine adducts are listed in Table 2. The visible spectral properties of the dyes were not altered by conversion to the sulfide derivatives (not all data shown). Due to the very strong absorption bands of the cyanine chromophore in the infrared region no differences between the reactive dyes and their cysteine conjugates were detectable. In fact, the cyanine absorption bands are so intense that the "amide 1" carbonyl stretching vibration of CY5.1-IA appears as a weak transition (1,654 cm^{-1}) next to a major cyanine band at 1,492 cm^{-1}. The polarity of the conjugates was significantly higher than the polarity of the iodoacetamide dyes allowing the straightforward demonstration of adduct formation by normal and reverse phase thin layer chromatography.

Sulfhydryl reactive cyanines have been recently used to modify -SH groups on proteins in situ. CY5.1-IA and CY5.7-IA, added as alcohol solutions, reacted with preparations of isolated rabbit skeletal muscle sarcoplasmic reticulum vesicles to induce a rapid Ca^{2+} release (23). Addition of low concentrations of the dyes (5–50 μM) was sufficient to trigger the calcium release. The labeling of particular proteins in the vesicle preparations was indicated by the occurrence of specific fluorescent bands separated by SDS polyacrylamide gel electrophoresis.

To further demonstrate the potential usefulness of these new reagents we have labeled a soluble protein, actin, with an iodoacetamido- cyanine dye. Actin is a major component of the cytoskeleton of most cells and functions by reversibly polymerizing to form filaments. Wang and Taylor have reported a method for labeling F-actin (filamentous form) specifically at Cys-374 with iodoacetamidofluorescein (29). This basic technique has been used with CY5.4-IA. In this case the dye was first adsorbed onto celite as an inert support since these dyes have limited water solubility. The recovered actin had a dye/protein ratio of 0.6, a fluorescence quantum yield of 0.13 and a maximum absorbance coefficient at 652 nm of 140,000. SDS-polyacrylamide gel electrophoresis of the product showed a single blue-colored band before staining which corresponded to the single protein band on the gel. The F- and G- states of the labeled actin had very similar excitation and emission spectra. The spectra of the labeled G-actin are shown in Figure 4. Spectra of free CY5.4-IA in the same buffer are included for comparison.

The cyanine-labeled actin had biochemical properties similar to those of the native protein. The actin analog formed filaments in vitro with a critical concentration for polymerization of 35 μg/ml. When injected into swiss 3T3 cells, the cyanine-labeled actin appeared to become associated with intracellular stress fibers in a manner similar to the incorporation of fluorescein labeled actin into stress fibers (1). Further characterization and biological applications of cyanine-labeled actin will be described elsewhere.

Cyanine and merocyanine polymethine dyes have been shown to be effective reagents for covalently labeling proteins. These dyes have high extinction coefficients, modest fluorescence yields, and moderate photostabilities. Their syntheses can be easily manipulated to produce dyes with absorption bands ranging from 500 nm to beyond 750 nm. This selection of fluorophores opens additional windows for multi-parameter fluorescence experiments. While the present iodoacetamido dyes are useful for the specific labeling of sulfhydryl groups of proteins, they are not ideal reagents for the general labeling of proteins such as antibodies. They have only moderate water solubility and most proteins have limited sulfhydryl content. Cyanine dyes with increased solubility in aqueous media and capable of reacting with protein amino groups are discussed in the following article.

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